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1 **Exploring the relationship between stress coping styles and sex, origin and**
2 **reproductive success, in Senegalese sole (*Solea senegalensis*) breeders in captivity**

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Highlights

- Senegalese sole reproductive success was not linked to stress coping styles.
- The sex of Senegalese sole was not linked to proactive or reactive coping styles.
- The origin, wild or 1st generation hatchery, of sole was not linked to coping styles.
- This nonaggressive social sole had equal opportunities in relation to coping styles.

35 **Abstract**

36 Individual animals commonly adopt different stress coping styles that have been shown
37 to impact reproductive success and differ between sexes (female/male) and origin
38 (wild/hatchery). Hatchery reared Senegalese sole (*Solea senegalensis*) exhibit a
39 behavioural reproductive dysfunction and a complete failure to spawn viable eggs. Hence,
40 the present study examined whether 1) reproductive success of Senegalese sole was
41 linked to coping styles and 2) behavioural differences exist in relation to sex or origin. A
42 total of 198 breeders held in two research institutions were submitted to three individual
43 tests (restraining, new environment and confinement) and one grouping test (risk taking).
44 In addition, a blood sample to quantify cortisol, glucose and lactate levels was obtained
45 from each individual after completing the individual tests. Senegalese sole breeders
46 showed individual differences in behaviour across the different behavioural tests that
47 were consistent with proactive and reactive coping styles traits. However, the most
48 striking result was that reproductive success, sex and origin of Senegalese sole was not
49 biased to any specific coping style. Indeed, the behavioural responses were similar and
50 consistent between fish grouped by reproductive success, sex and origin. This study
51 presented information that contrasts with different studies on dominant aggressive species
52 and indicated that social non-aggressive species such as Senegalese sole follow a
53 cooperative strategy that favours equal opportunities between stress coping styles and
54 sexes. Therefore, results suggest that maintaining both coping styles strategies are
55 fundamental for a sustainable breeder population approach.

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57 **Keywords:** *Solea senegalensis, coping styles, fitness, reproduction, sex differences*

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69 **Introduction**

70 Animals including fish when confronted with threatening or stressful situations have been
71 recognized to exhibit different behavioural responses (Koolhaas et al., 1999). These
72 patterns of behavioural responses have been defined as animal personalities (Dall et al.,
73 2004), behavioural syndromes (Sih et al., 2004) or when under challenging situations,
74 stress coping styles (Koolhaas et al., 1999). Different stress coping styles (hereafter SCS),
75 which is the selected term for the present study, have been documented in different taxa
76 of animals, such as birds, (Van Oers et al., 2005), mammals (Réale et al., 2009) and fishes
77 (Castanheira et al., 2015).

78 Stress coping styles represent a continuous axis of behavioural variation in
79 animals that ranges from two extremes: proactive to reactive (Koolhaas et al., 1999;
80 Øverli et al., 2007). When these two extremes are compared, proactive organisms have
81 been characterised to consistently present bold personalities, are highly active, are
82 motivated to take risk and to explore unfamiliar environments, are more aggressive and
83 have lower basal and post-stress glucocorticoids levels due to their higher hypothalamic-
84 pituitary-interrenal (HPI) axis activity (Koolhaas et al., 1999; Coopens et al., 2010;
85 Sørensen et al., 2013; Mittelbach et al., 2014). However, proactive fish were found to be
86 less flexible to environmental fluctuations and tended to follow routines (Sih et al., 2004;
87 Réale et al., 2009). On the other hand, reactive fish are less active, avoid taking risk and
88 exploring novel situations, avoid confrontation and usually pay more attention to external
89 stimuli and possess a higher capacity to adjust behaviour to novel situations (Koolhaas,
90 et al., 1999; Sih et al., 2004; Ruiz-Gomez et al., 2011). Such differences in behaviour
91 have been recognized to influence the overall fitness of fishes (Mittelbach et al., 2014;
92 Castanheira et al., 2015, Vargas et al., 2018).

93 Studies evaluating stress coping styles have documented that sex (female/male),
94 origin (wild/culture) and reproductive success were in some species biased to a specific
95 SCS. For instance, King et al. (2013) demonstrated that stickleback males (*Gasterosteus*
96 *aculeatus*) were more proactive, being significantly more active and prone to take risk
97 than females. Likewise, Ibarra-Zatarain et al. (2019) submitted the gilthead seabream
98 males and females to different coping styles tests and observed that males were more
99 active, produced lower glucocorticoids levels and took higher risk than females.
100 Regarding fish origin, Lepage et al. (2000) found that wild sea trout (*Salmo trutta*)
101 produced significantly lower plasma glucose and cortisol and showed elevated brain
102 levels of dopamine than domesticated trout after submitting fish to different stress tests.

103 Moreover, Huntingford and Adams (2005) observed that Atlantic salmon (*Salmo salar*)
104 hatched and kept in captivity were more prone to exhibit proactive behaviours and to take
105 higher risk, when submitted to novel situations, than wild individuals transferred to
106 captivity. Concerning reproduction, there is still an ongoing debate about if proactive or
107 reactive stress coping styles are factors that intrinsically influences mating and
108 reproductive success. In this context, proactive males of swordfish (*Xiphophorus helleri*)
109 (Royle et al., 2005), zebrafish (*Danio rerio*) (Ariyomo and Watt 2012; Vargas et al., 2018)
110 and gilthead seabream (*Sparus aurata*) (Ibarra-Zatarain et al., 2019) had higher
111 reproductive success and fertilized higher number of eggs than reactive males. However,
112 no relationship was reported between proactiveness and reproductive success in guppies
113 (*Poecilia reticulata*) (Piyapong et al., 2009) or mosquito fish (*Gambusia holbrooki*)
114 (Wilson et al., 2010). Thus, further investigations are needed to elucidate the mechanisms
115 and routes of action of stress coping styles towards reproduction, particularly in non-
116 aggressive species with social tendency, since most of studies evaluating SCS and
117 reproductive success have been performed in species that uses aggression as strategic
118 behaviour to achieve reproductive success and spawning (Godin and Dugatkin, 1996;
119 Cook et al., 2011; Ariyomo and Watt, 2012; Ibarra-Zatarain et al., 2019).

120 Senegalese sole (*Solea senegalensis*) is an important marine aquaculture species
121 that is commonly reared in intensive production systems in Southern European regions
122 (Morais et al., 2016). Senegalese sole is a benthonic, predominantly littoral fish species
123 found in sandy and muddy bottoms down to 100m and occasionally inhabit estuaries. The
124 species diet in the wild consists mainly of benthonic invertebrate, such as polychaetes,
125 molluscs and small crustaceans. However, the control of reproduction is a bottleneck that
126 is compromising the successful production in captivity of this species. Senegalese sole
127 captured from the wild and adapted to captive conditions spawn naturally in captivity
128 (Dinis et al., 1999; Anguis and Cañavate, 2005; Martín et al., 2014). Nonetheless,
129 broodstocks that were hatched and reared entirely in captivity do not produce viable eggs
130 (Guzman et al., 2009) and this has been attributed to a behavioural reproductive
131 dysfunction in the males that do not complete the courtship to fertilise the eggs (Mañanos
132 et al., 2007; Martín, et al., 2019). In this context, several studies have evaluated the
133 behavioural patterns of sole. For instance, Ibarra-Zatarain et al. (2016) showed that sole
134 exhibits well-defined proactive and reactive stress coping styles. Carazo et al. (2016)
135 found that sole have a complex courtship for mate selection and only spawn as pairs.
136 Martín et al. (2014) observed that these pairs showed fidelity within a spawning season

137 and between years and, furthermore, a large number of breeders did not participate in
138 spawning and may have been excluded by the established couples. Lastly, Fatsini et al.
139 (2017 and 2020) suggested that sole is not an aggressive species, but displays a
140 dominant/subordinate behaviour related to site preference or feeding areas. Considering
141 previous background, the present study investigated if reproductive success of Senegalese
142 sole was linked or not to proactive or reactive behaviours and established possible
143 individual behavioural differences according to sex (female / male) and origin (wild /
144 hatchery) of breeders. Results of the present investigation will provide a first insight on
145 the reproductive strategy of this species in relation to different stress coping styles.

146

147 **Ethic statement**

148 All experimental procedures on fish that formed part of this study was carried out in strict
149 accordance with the Spanish law (RD53/2013) and European regulations on animal
150 welfare (2010/63/UE and Federation of Laboratory Animal Science Associations,
151 FELASA), approved by the Animal Ethics Committee of the Institut de Recerca en
152 Tecnologies Agroalimentaries (IRTA) and in accordance to the Guidelines for the
153 treatment of animals in behavioural research and teaching (2012).

154

155 **Material and Methods**

156 **Fish maintenance**

157 A total of 198 Senegalese sole breeders, 59 held in IRTA (Sant Carles de la Rpita, Spain)
158 and 139 in the Spanish Oceanographic Institute, IEO (Santander, Spain), were used in the
159 present study. All sole breeders were tagged with a passive integrated transducer (PIT-
160 ID-100 Unique, Trovan-Zeus, Madrid, Spain) for individual identification.

161 Breeders from IRTA presented a mean weight of 1189 ± 50 g and were housed in
162 four 13 m³ rectangular tanks located in a greenhouse. To provide conditions similar to
163 those for the development of this fish species in nature, water was supplied to the tanks
164 with a recirculation system (IRTAmara® RAS system) that provided parameters similar
165 to those experienced in the species natural habitat with a temperature and oxygen levels
166 adjusted to 9 – 19°C (winter to summer) and 5 – 6 mg/L, respectively. Photoperiod was
167 natural ranging from light dark (L:D) 14:10 during summer to LD 10:14 in the winter.
168 Water temperature was 19°C and oxygen concentration was 6.0 mg/L during the
169 experimental period. Fish were hand-fed *ad libitum* in the morning (10:00 h) according
170 to the following regime: on Monday and Sunday balanced feed (Vitalis REPRO and LE-

171 7 ELITE line, Skretting Co.), on Wednesday cooked mussels (Sariego Intermares, Spain),
172 and on Tuesday and Friday, marine polychaetes (Topsy-Baits, Holland). One hour after
173 feeding, uneaten food was removed from tanks to maintain optimal physicochemical
174 water conditions.

175 Breeders from IEO presented a mean weight of $1357 \pm 28\text{g}$ and were housed in
176 four 14 m^3 rectangular tanks located in a building. The tanks were flow through with
177 simulated natural temperature ($11 - 20^\circ\text{C}$) and constant photoperiod (L:D) 14:10. Water
178 temperature was 19°C and oxygen level was 6 mg/L during the study period. The fish
179 were fed *ad libitum* in the morning according to the following regime: on Monday and
180 Friday cooked mussels (*Mytilus sp.*), and on Tuesday, Wednesday, Thursday and
181 Saturday fresh squid (*Loligo sp.*).

182

183 **Spawning and paternity analysis**

184 A passive egg collector was placed at the surface outflow of each spawning tank.
185 Spawning eggs were collected daily in the morning between 08:00 - 09:00 h and the
186 following parameters were determined and registered to determine the spawning quality:
187 a) volume of viable (floating) and unviable (sinking) eggs was determined using a 1 L
188 measuring cylinder, b) total fecundity, estimated by determining the number of eggs in a
189 5-ml sample and multiplying by the total volume of eggs and c) total fertilization rate by
190 counting the eggs with viable embryos in a sample of 50 eggs (by triplicate). Once the
191 quality of spawn was assessed, the fertilized eggs were transferred to a 30L vertical
192 incubators, with continuous water flow and aeration. After 36 - 48 h (at natural conditions
193 for the season, $19 - 23^\circ\text{C}$) hatching rate was calculated by counting the total estimated
194 hatched larvae / total number of eggs incubated. Three-day old larvae were collected for
195 the paternity analysis.

196 For paternity analysis, breeders from both centres were genotyped by analysing
197 DNA from caudal fin clips (see methodology by Martin et al., 2014). To assign paternity,
198 a sample of 10 larvae (3-day old) were collected from spawns and placed individually in
199 1.5 ml Eppendorf tubes filled with absolute ethanol after three washes in 96° alcohol. The
200 paternity assignment was carried out by GENEQUA (Facultad de Veterinaria de la
201 Universidad de Lugo, Lugo, Spain). The samples were genotyped using 6 microsatellites
202 loci isolated from the species (initially 4 microsatellites were used to determine paternity
203 and 2 extra microsatellites were only used for those samples that presented 3 or more
204 possible parents) in a single multiplex PCR (Martin et al., 2014; Fatsini et al., 2017). All

205 fish that were identified as parents from any spawn in the period 2013-2014 were
206 considered to have had reproductive success.

207

208 **Stress coping style tests**

209 The selected tests (restraining, new environment, confinement and risk taking) were
210 previously evaluated and confirmed as operational tests to characterize stress coping
211 styles in Senegalese sole (Ibarra-Zatarain et al., 2016). Overall, tests were carried on the
212 first and second week of October, in IRTA and IEO, respectively, and performed between
213 10:00 – 16:00 h in both locations. Tanks (dimension and colour), nets and other
214 instruments were of the same characteristics in both facilities to avoid possible
215 confounding of the results. Lastly, all tests were performed out of the breeding season in
216 order to reduce the influence of maturity status on fish behavioural responses.

217

218 **Individual coping style tests**

219 The first test, *restraining test*, consisted in capturing and maintaining a fish in the net out
220 of the water for 90 seconds and two variables were evaluated: Total Activity Time **NetAct**
221 (duration of fish movement in the net in seconds) and the Number of Escape Attempts
222 **NetEsc** (number of contortions or strong movements made by fish to escape in counts)
223 (Figure 1A). The definition of activity for this test was restricted to full body movements
224 made by fish to attempt escape from the net. The second test, *new environment test*, aimed
225 to evaluate the fish reaction to a novel environment. For this instance, fish were placed in
226 a 110 x 110 x 90 cm (width x length x depth) plastic tank (Figure 1B) and during a 5-
227 minutes period, two behavioural parameters were evaluated: First Activity time **NewLat**
228 and the Total Activity time **NewAct** (recorded in seconds). The third test, *confinement*
229 *test*, consisted of submitting the fish into a small plastic container 56 x 36 x 30 cm (width
230 x length x depth) (Figure 1C), that simulated a confined space, and for 5 minutes, two
231 behavioural parameters were evaluated: First Activity Time **ConLat** and the Total
232 Activity Time **ConAct** (recorded in seconds). The definition of activity, for both new
233 environment and confinement tests, was restricted to active locomotion or swimming. If
234 fish did not move during the 5-minute period, then 300 s was recorded and used for
235 statistical analysis (Farwell and McLaughlin, 2009; Ibarra-Zatarain et al., 2016).

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239 **Risk taking in groups test**

240 A single group coping style test, *risk-taking test*, was performed one month after
241 finalizing the three individual tests, to allow fish to recover. This test aimed to determine
242 fish capacity to cross from a known area (safe zone) to an unknown area (risky zone)
243 (Figure 1D). The test was performed in a 16 m³ tank 6.0 m x 3.0 m x 0.9 m (length x
244 width x depth), divided into two equal water volumes by a wood barrier. The safe zone
245 or shelter was isolated from light (2 lux; OSRAM DULUX 48 and 150W) and covered
246 with sand, to simulate natural conditions in the wild. On the contrary, the risky area was
247 more illuminated (11 lux on the surface of water; OSRAM DULUX 48 and 150W) and
248 the bottom of the tank was devoid of sand. Light intensity was adjusted in each area by
249 two external manual light dimmers. A window (30 cm width x 15 cm tall), which could
250 be opened from outside the tank, was at the base of the wooden barrier and when open
251 the sole could freely pass from the safe zone to explore the risky zone. This window was
252 at the centre of a PIT (passive integrated transducer) tag reading antenna (SQR series;
253 TROVAN-ZEUS, Madrid, Spain) that read the tag number of fish that passed through the
254 window, following criteria from Carter et al., 2013; Vargas et al., 2018 and validated by
255 Ibarra-Zatarain et al., 2016 for Senegalese sole).

256 Before the beginning of the test, breeders were submitted to a 24-hour acclimation
257 in the safe zone. After acclimation, the window was opened to begin the test and any fish
258 that crossed into the risky area during the following 24 hours were recorded by the PIT
259 tag antenna. Breeders were tested in groups of 10 individuals to avoid inducing stress due
260 to high stocking densities. The latency time of each organism to cross from one area to
261 another was recorded. A maximum time of 1440 min was assigned to fish that did not
262 cross during the 24-hour period of the test.

263

264 **Quantification of blood plasma cortisol, glucose and lactate**

265 Blood samples (0.5 ml) were extracted from the caudal vein of anesthetized fish (MS-
266 222; 100 ppm; Argent, USA,) to measure cortisol, lactate and glucose concentrations.
267 Blood extraction was performed approximately 40 minutes after completing individual
268 tests. To avoid blood coagulation, a solution of 10 µl sodium heparin (5%, 25.000 UI;
269 HOSPIRA) and 15 µl aprotinin (from bovine lung; 0.9% NaCl, 0.9% benzyl alcohol and
270 1.7 mg of protein; SIGMA) was placed inside the 1.5 ml plastic tubes (Eppendorfs), while
271 syringes and needles were coated with heparin. Blood samples were centrifuged at 3000
272 G and 4°C during 15 min (ThermoScientific centrifuge, M23i; Thermo rotor AM 2.18;

273 24 x 1.5 ml) and plasma supernatant was removed and stored in triplicates at -80°C prior
274 to analysis (Ibarra-Zatarain et al., 2016). Cortisol level was measured with a commercial
275 ELISA kit (Range of detection: 0 - 800 ng/mL; DEMEDITEC, Kiel-Wellsee, Germany),
276 by means of a competitive reaction with a conjugated binding ligand, whereas glucose
277 and lactate concentrations were measured by means of a commercial enzymatic
278 colorimetric kit (SPINREACT, Gerona, Spain) and read by a spectrophotometer (Infinite
279 M-200; TECAN, Switzerland) at 23°C and 505 nm, following the methodology validated
280 by Ibarra-Zatarain et al. (2016) for this fish species.

281

282 **Statistical analysis**

283 All statistical analyses were performed using PASW 20 software for Windows. Normality
284 of data was checked through a Kolmogorov-Smirnov test with Lilliefors correction. Two
285 approaches were used to characterise coping styles of Senegalese sole by having a
286 behavioural composite and comparing it with the physiological state, reproductive
287 success, sex and origin of individuals. The first approach considered the performance of
288 fish in each individual test and examined how this was related to reproductive success,
289 sex, origin and holding institution. The second approach considered if components
290 collectively were related to the performance of individual fish in all of the SCS tests were
291 related to reproductive success, sex, origin and holding institution.

292 First approach consisted in evaluating the variables measured in each individual
293 coping style test (NetAct and NetEsc for the restraining test, NewAct and NewLat for the
294 new environment test and ConAct and ConLat for the confinement test) with three
295 successive PCA (one per test). Then, the three Principal Component Scores resulting from
296 these PCA's (hereafter defined as *restraining-PCS1*, *new environment-PCS2* and
297 *confinement-PCS3*) were used as single composite score that represented the individual
298 behaviour index for each individual test (Budaev, 1997; Wilson and Godin, 2009) and
299 validated for this fish species by Ibarra-Zatarain et al. (2016). The second approach
300 consisted in assessing the six variables (two per test) and glucocorticoids hormones
301 (cortisol, glucose and lactate) into a single PCA and two components were generated
302 (*PC1-global* and *PC2-global*). A Bartlett's test of sphericity and a Kaiser-Meyer-Olkin
303 test was performed to test for variable and sample adequacy to the PCA analysis.
304 Correlations among coping styles variables of fish grouped according to their
305 reproductive success, sex, origin and research institute were analysed by Point-biserial
306 correlations, an extension of Pearson analysis for dichotomous variables.

307 Once the principal component scores of the two approaches were generated, two General
308 Multivariate Linear Models (GLMM) were performed: **i)** on the *restraining-PCS1*, *new*
309 *environment-PCS2*, *confinement-PCS3* and cortisol, glucose and lactate concentrations
310 and **ii)** on *PC1-global* and *PC2-global*. The GLMM was performed to identify possible
311 significant differences between fish with different reproductive success (spawned / not
312 spawned), between sex (female / male), origin (wild / hatchery), research institutes (IRTA
313 / IEO) and results obtained in the risk-taking test (crossed / not crossed). A Kolmogorov-
314 Smirnov test (KS-test), with Fisher's Z-test, was performed to analyse frequency
315 distribution of behaviours in breeders grouped according to their reproductive success,
316 sex, origin and groups. A logistic regression analysis, with a Fisher's exact test, was
317 performed to establish if the latency time to move in the new environment (NewLat) and
318 in confinement (ConLat) tests were correlated with the fish that crossed and did not cross
319 (yes / no variables) in the risky area. Lastly, a Chi-square test (X^2 -test) was executed to
320 establish significant differences in the proportion of fish that crossed in the risk-taking
321 test versus those that did not cross, between the fish separated by reproduction success,
322 sex and origin. Values are presented as means \pm standard deviation. Statistical differences
323 were established when $P < 0.05$ for all analysis.

324

325 **Results**

326 **Statistical approaches to analyse stress coping styles**

327 The first statistical approach showed that **NetEsc**, **NewLat** and **ConLat** were the
328 variables that explained the highest variance in the 3 individual tests, 72.58% of the
329 *restraining-PCS1*, 69.27% of the *new environment-PCS2*, 62.26% of the *confinement-*
330 *PCS3*, respectively, and presented eigenvalues greater than 1. Moreover, the Pearson's
331 correlation analysis showed that *restraining-PCS1* was significantly and negatively
332 correlated with *new environment-PCS2* ($R = -0.301$, $P < 0.001$) and *confinement-PCS3*
333 ($R = -0.341$, $P < 0.001$), suggesting that those fish with more escape attempts (higher
334 scores) started to explore the new environment and resumed activity in confinement
335 earlier (lower scores). In addition, *new environment-PCS2* was positively correlated with
336 *confinement-PCS3* ($R = 0.412$, $P < 0.001$). Whilst the second statistical approach
337 (considering all variables together), showed that **NetAct** and **NewAct** explained the
338 higher variance (42.8%). However, these two factors were not significantly correlated
339 (Pearson, $P > 0.05$).

340

341 **Individual and group coping style characterization**

342 In the individual tests, Senegalese sole ($n = 198$) showed a high behavioural variability
343 in restraining (total activity: min = 0 to max = 80 sec, CV = 93.4%; escape attempts: min
344 = 0 to max = 49, CV = 134.7%), new environment (latency: min = 1 to max = 300 sec,
345 CV = 143.4%; total activity: min = 0 to max = 227 sec, CV = 130.50%) and confinement
346 (latency: min = 1 to max = 300 sec, CV = 203.9%; total activity: min = 0 to max = 132
347 sec, CV = 132.8%) tests. Minimum and maximum values of these variables showed two
348 extremes of coping styles, proactiveness and reactiveness. In the grouping test (Figure 3),
349 29 individuals (IRTA = 17, IEO = 12) crossed from the safe to the risk zone and 169 did
350 not cross (IRTA = 42, IEO = 127), and the chi-square test showed that risk taking (fish
351 that crossed or did not cross) and institution (IRTA or IEO) were dependent variables
352 (chi-square $X^2 = 13.496$, $F_1 = 12.366$, $P < 0.001$), suggesting that sole held in IRTA took
353 higher risk than those held in IEO. Furthermore, the first statistical approach
354 demonstrated that sole that crossed had significant higher escape attempts in the
355 *restraining-PCS1* (GLMM, $F_{173} = 3.71$, $P < 0.05$) than fish that did not cross. However,
356 no statistical differences between fish that crossed and did not cross were found in the
357 *new environment-PCS2* (GLMM, $F_{173} = 0.143$, $P = 0.521$), *confinement-PCS3* (GLMM,
358 $F_{173} = 1.15$, $P = 0.285$), cortisol (GLMM, $F_{173} = 0.416$, $P = 0.520$), glucose (GLMM, F_{173}
359 $= 1.91$, $P = 0.169$) and lactate (GLMM, $F_{173} = 0.934$, $P = 0.335$) levels (Figure 2). Fish
360 that crossed and did not crossed did not show statistical differences when considering the
361 second statistical approach, neither for *PC1-global* (GLMM, $F_{173} = 0.2.878$, $P = 0.092$)
362 nor for *PC2-global* (GLMM, $F_{173} = 0.063$, $P = 0.802$). Latency time to cross was
363 statistically linearly correlated with *confinement-PCS3* ($R = 0.535$, $F_{196} = 8.432$, $P <$
364 0.001), but not with either *restraining-PCS1* ($R = 0.254$, $F_{196} = 3.947$, $P < 0.05$), *new*
365 *environment-PCS2* ($R = 0.321$, $F_{196} = 1.158$, $P < 0.05$). No significant correlations
366 (Pearson, $P > 0.05$) were detected between fish that crossed and did not cross regarding
367 the latency time to move in the new environment and confinement tests.

368

369 **Reproductive success and coping styles**

370 Behavioural responses of fish that successfully spawned ($n = 54$) were similar to those
371 that did not spawn ($n = 144$) in the three individual tests (Table 1). Further, the GLMM
372 showed that those fish that successfully spawned behaved similarly to those that did not
373 spawn and no differences were detected when analyse their components with the first
374 approach (*restraining-PCS1* GLMM, $F_{173} = 1.45$, $P = 0.230$, *new environment-PCS2*

375 GLMM, $F_{173} = 0.593$, $P = 0.442$ and confinement-PCS3 GLMM, $F_{173} = 0.483$, $P =$
376 0.490) and second approach (GLMM, PC1-global $F_{184} = 0.282$, $P = 0.596$ and PC2-
377 global $F_{184} = 0.193$, $P = 0.661$). Moreover, successful and unsuccessful breeders showed
378 similar frequency distributions (first approach KS-test restraining-PCS1 $P = 0.425$, new
379 environment-PCS2 $P = 0.598$ and confinement-PCS3 $P = 0.822$; second approach KS-
380 test PC1-global $P = 0.493$ and PC2-global $P = 0.982$). In addition, blood parameters were
381 similar in fish of both groups (GLMM, cortisol $F_{173} = 0.001$, $P = 0.999$, glucose $F_{173} =$
382 0.021 , $P = 0.884$ and lactate $F_{173} = 0.011$, $P = 0.916$). Lastly, the chi-square test showed
383 that risk taking (fish that crossed or did not cross) and reproductive success (fish that
384 reproduce or did not reproduce) were independent variables ($X^2 = 0.742$, $F_1 = 0.779$, P
385 $= 0.268$), suggesting that reproduction of sole is not related to coping styles (approach
386 one Figure 4A; approach two Figure 5A).

387

388 **Sex and coping styles**

389 Males ($n = 88$) and females ($n = 110$) behaved similarly in the individual stress coping
390 styles tests (Table 1). The first statistical approach showed that males and females
391 behaved similar in the *new environment-PCS2* (GLMM, $F_{173} = 0.013$, $P = 0.909$) and
392 *confinement-PCS3* (GLMM, $F_{173} = 0.267$, $P = 0.267$) (Figure 4B). Indeed, the KS-test
393 showed that these two components had similar frequency distributions in both groups (P
394 $= 0.790$ and $P = 0.837$, respectively). Likewise, the second approach (Figure 5B) showed
395 no statistical differences between behaviours of males and females (GLMM, PC1-global
396 $F_{184} = 0.029$, $P = 0.864$ and GLMM, PC2-global $F_{184} = 0.070$, $P = 0.792$) and between
397 their distributions (KS-test, PC1-global $P = 0.646$ and PC2-global $P = 0.287$). Blood
398 parameters were not significantly different between males and females (GLMM, cortisol
399 $F_{173} = 2.09$, $P = 0.150$, glucose $F_{173} = 0.606$, $P = 0.437$ and lactate $F_{173} = 2.35$, $P = 0.127$).
400 Besides, the X^2 test showed that males and females did not differ in their risk taking (X^2
401 $= 1.584$, $F_1 = 1.573$, $P = 0.146$).

402

403 **Origin and coping styles**

404 Behaviours of hatchery breeders ($n = 100$) were similar to wild breeders ($n = 98$) in
405 *restraining-PCS1* (GLMM, $F_{173} = 3.61$, $P = 0.060$), *new environment-PCS2* (GLMM,
406 $F_{173} = 1.37$, $P = 0.243$) and *confinement-PCS3* (GLMM, $F_{173} = 0.220$, $P = 0.883$) analysed
407 with the first approach (Figure 4C; Table 1). Moreover, both groups presented highly
408 similar distributions for the three PCs (KS-test, PCS1 $P = 0.501$, PCS2 $P = 0.268$ and

409 PCS3 $P = 0.311$). The second approach showed no statistical differences and similar
410 frequency distributions between hatchery and wild fish (GLMM, PC1-global $F_{184} =$
411 0.003 , $P = 0.959$ and PC2-global $F_{184} = 0.863$, $P = 0.354$; KS-test $P = 0.870$ and $P =$
412 0.483 , respectively) (Figure 5C). Likewise, no statistical differences were detected in the
413 risk-taking test between hatchery and wild breeders ($X^2 = 3.063$, $F_1 = 3.110$, $P = 0.065$).

414

415 **Sole coping styles by research institutions**

416 IEO breeders ($n = 139$) exhibited significantly higher scores for *restraining-PCSI*
417 (GLMM, $F_{173} = 5.21$, $P = 0.024$) (Figure 4D) and produced less glucose and lactate levels
418 (GLMM, $F_{173} = 53.91$, $P < 0.001$; $F_{173} = 49.74$, $P < 0.001$, respectively) than breeders from
419 IRTA ($n = 59$). Nevertheless, the *new environment-PCS2*, *confinement-PCS3* and cortisol
420 were not significantly different (GLMM, $F_{173} = 0.712$, $P = 0.400$, $F_{173} = 0.257$, $P = 0.613$
421 and $F_{173} = 0.812$, $P = 0.369$, respectively). The KS-test also showed different behavioural
422 distributions between both groups for *restraining-PCSI* ($P = 0.041$) and *confinement-*
423 *PCS3* ($P = 0.049$). The second approach (Figure 5D) showed significant differences
424 between fish from IEO and IRTA and between their distributions in *PC2-global* (GLMM,
425 $F_{173} = 6.178$, $P = 0.010$; KS-test $P = 0.001$), but not in *PC1-global* (GLMM, $F_{173} = 1.969$,
426 $P = 0.162$; KS-test $P = 0.002$).

427

428 **Discussion**

429 **Behavioural characterization of Senegalese sole breeders**

430 Overall, we have described individual differences in behaviour between Senegalese sole
431 breeders and classified individuals as proactive and reactive. Senegalese sole with high
432 activity, low latency to explore novel situations and low glucocorticoids, glucose and
433 lactate blood plasma levels were defined as proactive, whilst sole that exhibited lower
434 activity, high latency to start exploration in a new environment and high glucocorticoids
435 and glucose and lactate blood plasma levels were defined as reactive, in accordance with
436 Ibarra-Zatarain et al. (2016) for this species. Moreover, it was observed that individuals
437 with higher number of escape attempts (high score in *restraining-PCSI*) started to explore
438 the new environment and resumed activity in confinement earlier (low score in *new*
439 *environment-PCS2* and *confinement-PCS3*) confirming hence the existence of
440 behavioural syndromes in adult individuals of this species. These behavioural criteria, to
441 differentiate the proactive from reactive Senegalese sole, agrees with previous studies
442 performed with this (Silva et al., 2010; Ibarra-Zatarain et al., 2016; Fatsini et al., 2017;

443 Fatsini et al., 2019; Ibarra-Zatarain et al., 2020) and other fish species (Koolhaas et al.,
444 1999; Brelin et al., 2005; Farwell and McLaughlin, 2009; Castanheira et al., 2015).

445

446 **Sex and stress coping styles**

447 Several fish models have suggested that males are prone to present proactive styles, while
448 females are usually associated to reactivity (Godin and Dugatkin, 1996; Candolin,
449 1999; Harris et al., 2010; Ariyomo and Watt, 2012, King et al., 2013; Mamuneas et al.,
450 2014). These interpretations are based on the observation that males had higher overall
451 activity, foraged more in risky situations, resumed activity earlier than females after a
452 stressful situation and made faster decisions towards food reward in unknown contexts
453 (Harris et al., 2010; Schuett et al., 2010; King et al., 2013). In the present study,
454 Senegalese sole males and females exhibited similar coping abilities to stress, with an
455 exception in the number of escape attempts (*restraining-PCS1*), in which males attempted
456 to escape more than females. Nevertheless, it is important to notice that females were
457 significantly heavier than males and this factor possibly reduced the ability of females to
458 attempt escaping from the net, although no significant correlations were observed
459 between weight and coping style responses. This result is contrary to several hypotheses
460 that have suggested that males and females differ in their personality and in their strategy
461 to counteract stressful situations (Harris et al., 2010; Ariyomo and Watt, 2012, King et
462 al., 2013). Schuett et al. (2010) proposed that behaviour consistently differs between
463 sexes because “*the competition and requirements for accessing to reproduction leads to*
464 *greater variance in males than of females*”. Thus, the hypotheses suggest that males are
465 expected to maximize their fitness by taking higher risks, dominating other males and
466 foraging more distance to increase their opportunities to reproduce and to provide their
467 genetic charge to fry, whereas females give advantage to a longer life-span to maximize
468 their reproductive opportunities, hence, they reduced foraging and risk taking
469 (Andersson, 1994; Piyapong et al., 2009; Harris et al., 2010; Schuett et al. 2010; King et
470 al., 2013). Even in situations where males cannot dominate, males use strategies of
471 “sneaking” to reproduce with as many females as possible. However, in Senegalese sole
472 this appears to not be the case and seems to be more related to “a cooperation system”
473 where coping abilities to stressful situations offer no advantage to either sex and this
474 similitude in behavioural patterns might help both sole, males and females, to reduce
475 competition, defend territories, avoid injuries of individuals or increase breeding success
476 (see Taborsky, 1994; Stiver et al., 2005; Le Vin et al., 2011). Furthermore, Senegalese

477 sole is a social species and their mating systems is characterized by the formation of single
478 monogamous pairs that exhibit fidelity (Martin et al., 2014; Carazo et al., 2016; Martin et
479 al., 2019). Therefore, in a species with these characteristics and where the opportunities
480 for reproductive success are similar for the two sexes the hypothesis of competition and
481 requirements for accessing to reproduction leads to greater variance in males than of
482 females does not apply. Thus, the absence of correlations between sex and SCS in sole is
483 in accordance to the observation that males and females have similar variation in
484 reproduction and do not compete to attract many mates as is witnessed in other fish
485 species, which uses proactiveness/aggression as behavioural strategy to increase
486 opportunities to find mates and successfully reproduce, as observed in zebrafish (Vargas
487 et al., 2018) and seabream (Ibarra-Zatarain et al., 2019).

488

489 **Origin and stress coping styles**

490 It has been hypothesized that fish domestication may have profound effects on behaviour
491 and adaptation (Huntingford, 2004; Robinson and Rowland, 2005). In the present study,
492 hatchery and wild breeders showed similar behavioural responses and no significant
493 differences were detected between their behavioural scores and their glucocorticoids
494 levels, but close to significance as they present a clear trend. In addition, morphometric
495 parameters were not significantly correlated with stress coping responses. The lack of
496 significant behavioural differences between wild and hatchery-reared Senegalese sole
497 may be attributed to life experience of individuals, to the fish capacity of adaptation to
498 captivity or that the Senegalese sole were the first-generation breed in captivity with little
499 advance in the domestication process (Huntingford, 2004; Adriaenssens and Johnson,
500 2011). Nonetheless, hatchery breeders presented a higher, but not statistically different,
501 activity in the individual tests (restraining “NetAct”, new environment “NewAct” and
502 confinement “ContAct”) and in their risk-taking capacity in comparison of wild
503 individuals. Therefore, this low, but detectable, variability in behaviours between wild
504 and hatchery-reared fish might be considered as the first consequence of domestication
505 and genetic changes, which played a fundamental role on fish personality modelling
506 (Dingemanse et al., 2012). Similar observations and tendencies, in overall activity and
507 risk taking to those observed in the present study have been reported in other fish species,
508 such as zebrafish *Danio rerio* (Robinson and Rowland, 2005), rainbow trout
509 *Oncorhynchus mykiss* (Biro et al., 2006), brown trout *Salmo trutta* (Adriaenssens and
510 Johnson, 2011), seabass *Dicentrarchus labrax* (Benhaïm et al., 2013) and Atlantic salmon

511 *Salmo salar* (Metcalf et al., 2003). In addition, Huntingford and Adams (2005) reviewed
512 that hatchery-reared salmonids regularly tended to be proactive, more aggressive and took
513 higher risk when foraging than wild specimens. In captivity, fish are involved into a
514 constant selection for improving growth, promoting disease resistance and increasing
515 overall performance and cognition (Huntingford, 2004; Huntingford and Adams, 2005;
516 Benhaïm et al., 2013). Nevertheless, it is worth to consider that these slight behavioural
517 differences in activity and in risk taking between wild and hatchery-reared fish can be the
518 reflection of a pre-existing genetic variation between both strains, which are innate and
519 independent of domestication, but related to different coping style strategies. However,
520 the fish in the present study are the first generation in captivity and more studies are
521 necessary on future generations of this fish species to confirm previous speculations.

522

523 **Reproduction success and stress coping styles**

524 The aim of the investigation was to evaluate if SCS and reproduction were correlated in
525 Senegalese sole, as have been observed in different studies performed in different taxa,
526 such in mammals (red squirrels *Tamiasciurus hudsonicus* (Boon et al., 2007), bighorn
527 sheep rams *Ovis candensis* (Réale et al., 2009)), birds (ural owl *Strix uralensis*
528 (Konttinen et al., 2009), zebra finches *Taeniopygia guttate* (Schuett et al., 2011)), insects
529 (fishing spider *Dolomedes fimbriatus* (Arnqvist and Henriksson, 1997)), lizards (Indian
530 rock agama *Psammophilus dorsalis* (Batabyal and Thaker, 2018)) and fish (swordfish
531 *Xiphophorus helleri* (Royle et al., 2005), zebrafish *Danio rerio* (Vargas et al., 2018),
532 gilthead seabream *Sparus aurata* (Ibarra-Zatarain et al., 2019)). Nevertheless, the coping
533 strategies exhibited by successful and unsuccessful Senegalese sole breeders were similar
534 and no behavioural differences were detected. As initially commented, most of studies
535 that evaluated the correlation between coping styles and reproduction have mainly
536 highlighted aggression as a behavioural tactic linked to proactiveness or boldness, which
537 is used by individuals to increase reproductive success. Even more, Smith and Blumstein
538 (2008) reported that behavioural traits are positively linked to mating success and
539 commented that aggressive and proactive individuals are favoured during reproduction.
540 According to Fatsini et al. (2017), Senegalese sole is a social and non-aggressive specie,
541 but exhibits a dominance / subordination behaviour without aggression or fights amongst
542 conspecifics. In this context, a possible hypothesis could be that social animals tend to
543 create some forms of cooperation to make their subsistence successful by promoting
544 synchronised behaviours to counteract harmful situations, create coalitions and share

545 reproduction. This theory is reinforced by different reviews that have demonstrated that
546 animals living in social dynamic systems provide benefits to individuals in terms of
547 evolution, adaptation, reduced predation risk, acquire resources, genetic and fitness (Silk,
548 2007; Pike et al., 2008; Maruska and Fernald, 2013, Fernald, 2015). In terms of
549 reproduction, several studies have suggested that dominant and aggressive individuals
550 monopolize spawning. For instance, Vargas et al. (2018) and Ibarra-Zatarain et al. (2019)
551 described that males and females of zebrafish and gilthead seabream, respectively,
552 utilized aggression to dominate reactive individuals during spawn. Therefore, authors
553 found that SCS were significantly linked with reproductive success. However, the
554 opposite is detected in social animals living in groups, where frequently a change of
555 leadership can be observed (meaning that subordinate individuals can become dominant
556 and vice-versa). Thus, opportunities of reproduction are similar amongst individuals, as
557 it has been reported in studies performed in macaques *Macaca sylvanus* (Kuester et al.,
558 1995), rabbits *Oryctolagus cuniculus* (Von Holst et al., 2002), grey wolves *Canis lupus*
559 (Peterson et al., 2002), zebra *Equus burchelli* (Fischhoff et al., 2009), degus *Octodon*
560 *degus* (Wey et al., 2013) and cichlid fish *Neolamprologus pulcher* (Dey et al., 2015).
561 Hence, it is possible to conclude that reproductive tactics of Senegalese sole were in line
562 with tactics performed by social species (Fatsini et al., 2017) and are less influenced by
563 proactive-reactive traits, contrary to aggressive species. Although further studies are
564 needed to confirm the hypothesis that all Senegalese sole fish have the same opportunity
565 for reproduction and spawning, independently from their SCS response.

566

567 **Conclusions**

568 Overall results demonstrated that Senegalese sole exhibit defined stress coping styles.
569 However, the key results were to demonstrate that proactive or reactive patterns were not
570 significantly related to reproductive success, sex and origin of Senegalese sole. The
571 present study is particularly important, since Senegalese sole with proactive or reactive
572 traits have similar opportunities of reproduction, therefore, it is possible to suggest that
573 conserving both coping strategies likely allows this species to improve the ability of
574 individuals to maximize their opportunities for adaptation and subsistence of their future
575 progeny to different environmental situations. Moreover, the present study demonstrated
576 that reproductive dysfunctions of Senegalese sole appeared not to be biased to proactive
577 or reactive styles, as was initially thought, but is more related to a strategy of social
578 animals living in groups.

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586

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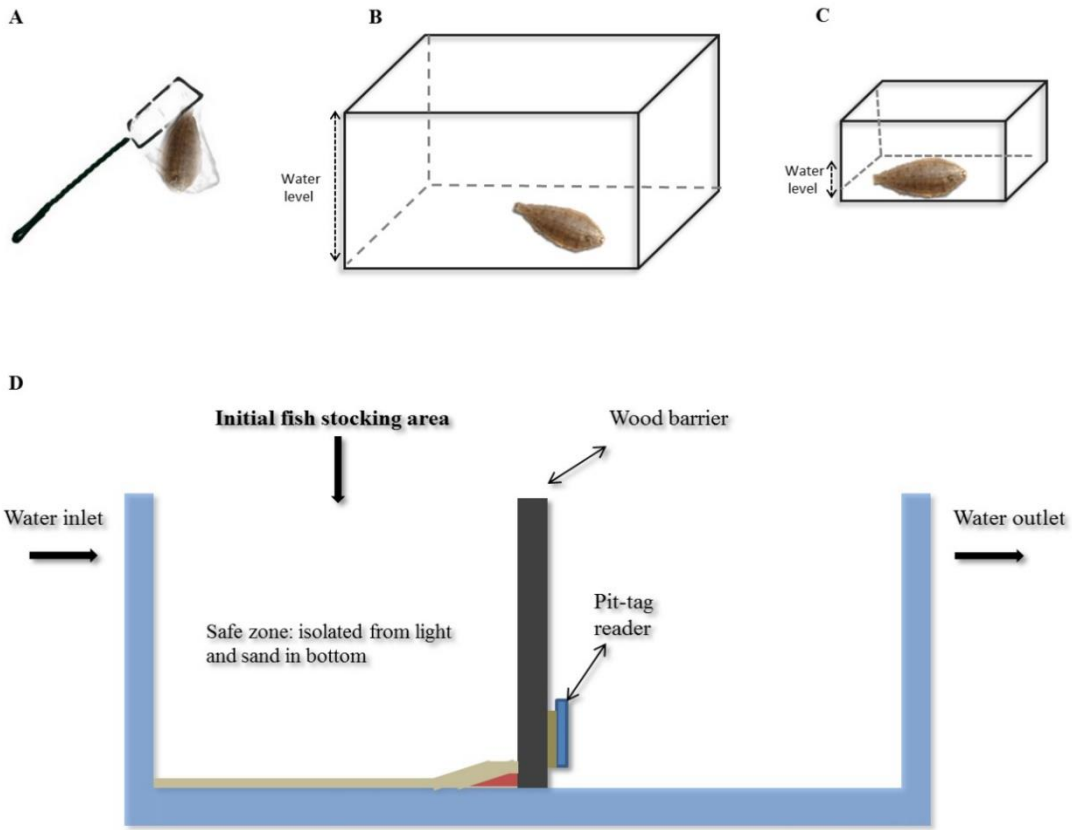
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1 **Figures**



19 **Figure 1.** Description of equipment used to perform the coping styles tests on Senegalese
20 sole breeders. **A=** Restraining test; **B=** Novel environment test; **C=** Confinement test; **D=**
21 Risk taking test

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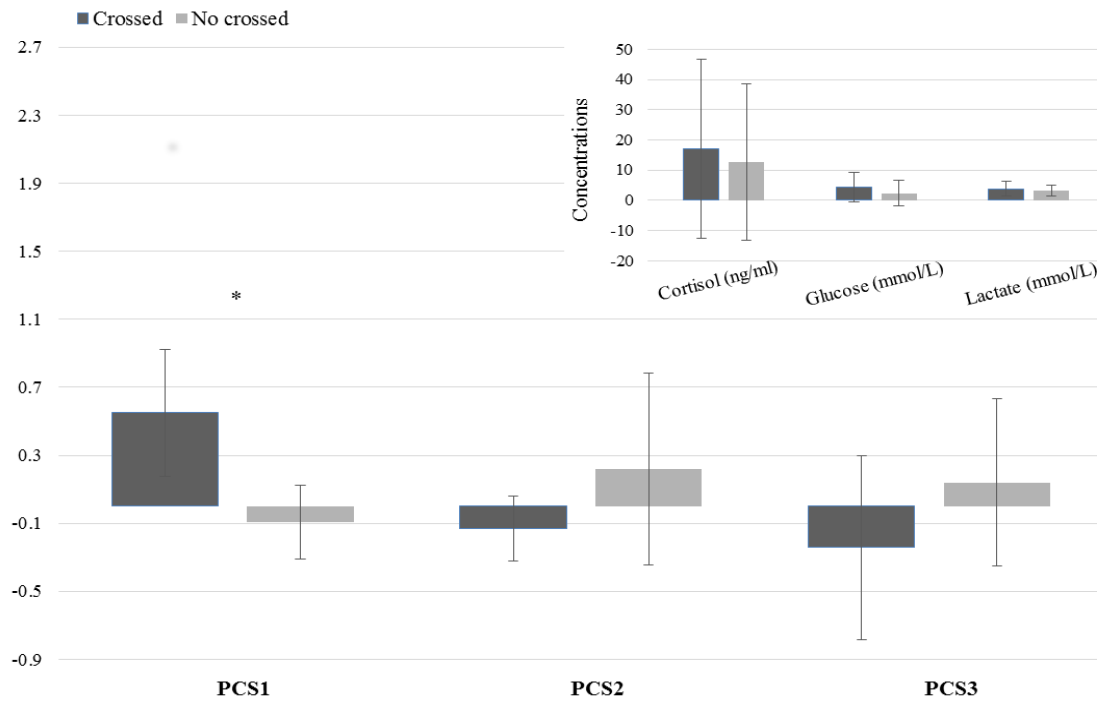


Figure 2. PCS and glucocorticoids concentrations differences between the fish that successfully crossed and those that did not cross. * Indicates significant differences

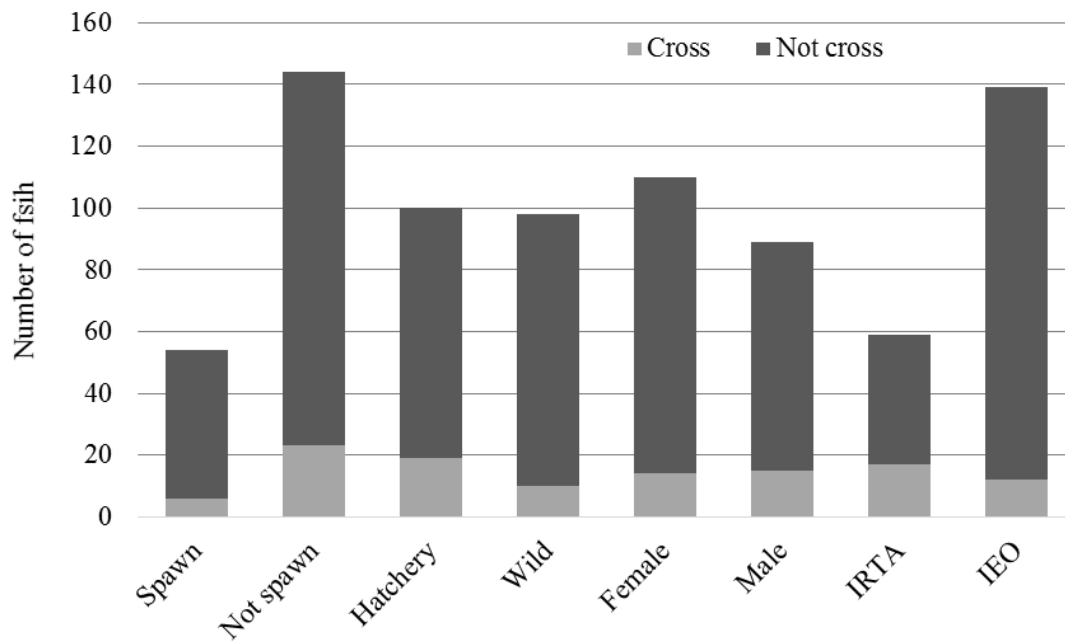


Figure 3. Number of fish that successfully crossed (light grey) and did not cross (dark grey) in the risk-taking test, grouped by reproductive success, origin, sex, and institute. * Indicates significant differences

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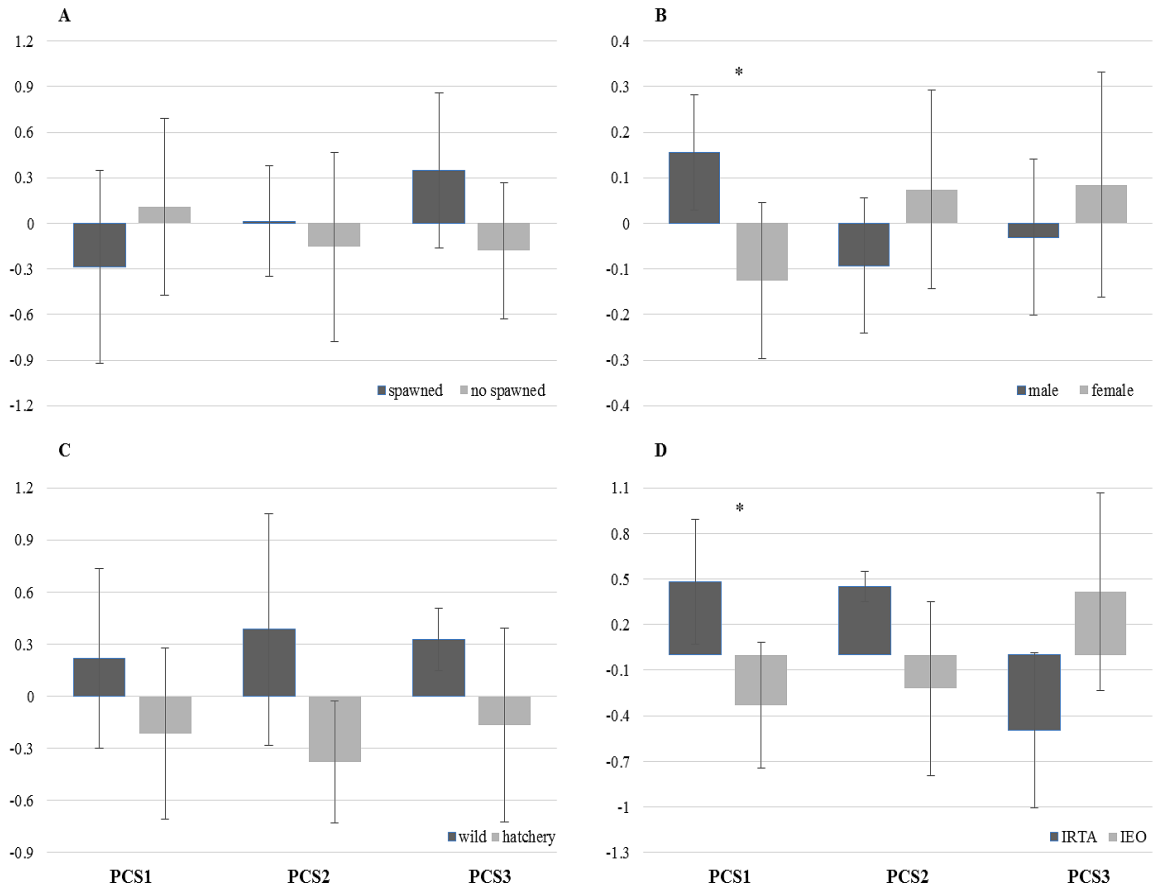


Figure 4. Comparison of principal component scores of sole breeders calculated from the restraining (PCS1), new environment (PCS2) and confinement (PCS3). Graphics split by sex (A), origin (B), spawning success (C) and research institution (D). * indicates significant differences between groups of fish within a PCS ($P < 0.05$).

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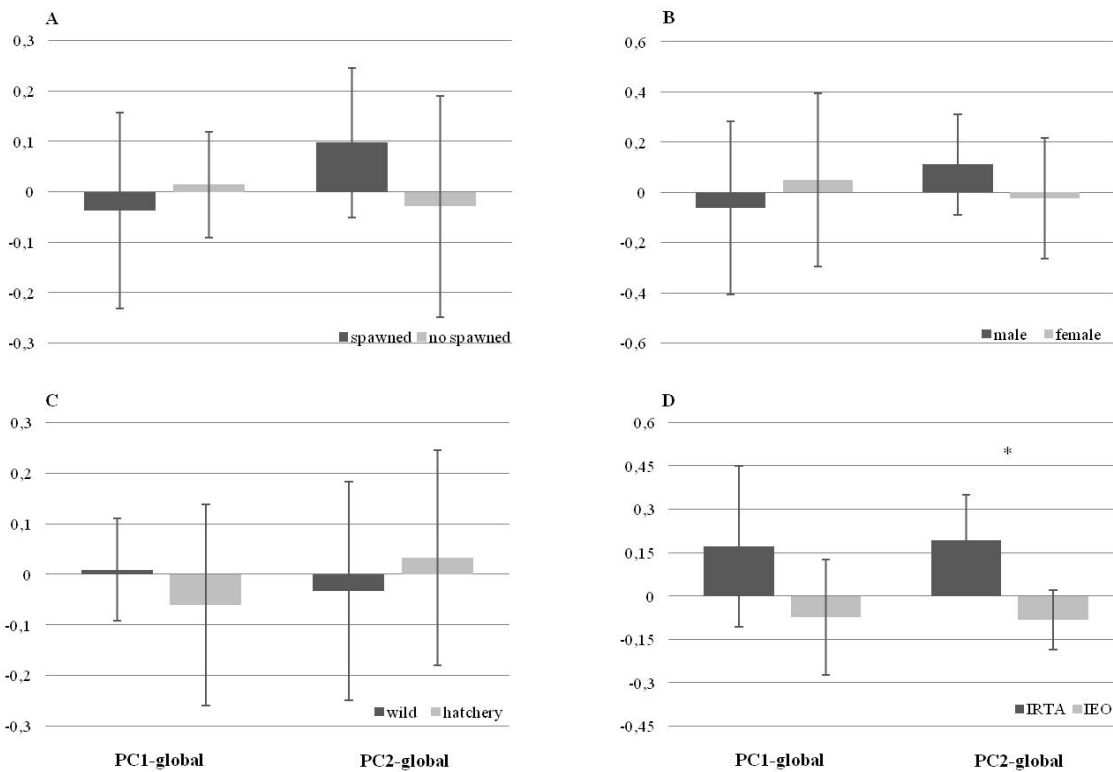
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155 **Figure 5.** Comparison of principal component scores of sole breeders calculated from the
156 second approach considering all six variables and glucocorticoids levels. Graphics split
157 by sex (A), origin (B), spawning success (C) and group (D). * Indicates significant
158 differences between groups of fish within a PCS ($P < 0.05$).

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1 **Table 1.** Comparison between sexes, origin and spawning success for fish morphology, variables for test 1-3 and biochemical quantifications
2 (means \pm sem). Numbers in parenthesis correspond to the number of animals in each group. Capital superscript letters designated significant
3 differences between fish weight and length (resulted from GLMM). Cortisol is expressed in ng/mL, glucose and lactate in mmol/L

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Tests	Variable	Female (110)	Male (88)	Hatchery (100)	Wild (98)	Spawn (54)	No spawn (144)	IRTA (59)	IEO (139)
Morphology	Weight	1391 \pm 37 ^A	1200 \pm 29 ^B	1240.2 \pm 28.6 ^A	1374.8 \pm 41.3 ^B	1350.0 \pm 55	1290.7 \pm 28.1	1190 \pm 50	1357 \pm 29
	Length	47.0 \pm 0.6	47.2 \pm 0.5	44.0 \pm 0.4 ^A	50.4 \pm 0.5 ^B	50.1 \pm 0.7 ^A	46.0 \pm 0.4 ^B	45.8 \pm 0.6	47.7 \pm 0.5
Netting the fish	NetEsc	17 \pm 1.5	21.6 \pm 2.1	17.3 \pm 1.8	20.7 \pm 1.2	20.7 \pm 2.1	18.3 \pm 1.5	16.4 \pm 1.7	20.1 \pm 1.6
	NetAct	4.6 \pm 0.6	6.2 \pm 0.9	5.7 \pm 0.8	5.0 \pm 0.6	4.6 \pm 0.8	5.6 \pm 0.6	7.4 \pm 1.1	4.5 \pm 0.5
New environment	NewLat	73.3 \pm 11.0	66.4 \pm 11.6	73.3 \pm 11.1	67.0 \pm 11.3	69.2 \pm 15.0	70.5 \pm 9.3	98.3 \pm 14.5	58.3 \pm 9.3
	NewAct	19.2 \pm 2.2	25.1 \pm 3.2	24.3 \pm 2.9	19.4 \pm 2.5	21.1 \pm 3.7	22.2 \pm 2.2	26.4 \pm 3.9	20.2 \pm 2.1
Confinement	ConLat	58.9 \pm 10.0	49.1 \pm 10.3	53.0 \pm 10.0	56.1 \pm 10.4	50.4 \pm 13.2	56.1 \pm 8.5	66.1 \pm 12.2	49.6 \pm 8.8
	ConAct	21.4 \pm 3.0	18.7 \pm 2.8	22.8 \pm 2.8	17.5 \pm 2.9	17.0 \pm 3.1	21.5 \pm 2.5	27.9 \pm 3.7	16.9 \pm 2.4
Risk taking	Cross	14	15	19	10	6	23		
	Not cross	96	74	81	88	48	121		
Blood analysis	Cortisol	11.8 \pm 2.7	15.1 \pm 3.0	15.2 \pm 2.6	11.3 \pm 3.0	12.2 \pm 4.4	13.6 \pm 2.2	16.7 \pm 5.2	11.7 \pm 1.8
	Glucose	3.1 \pm 0.2	3.2 \pm 0.2	3.3 \pm 0.2	3.0 \pm 0.2	3.1 \pm 0.2	3.1 \pm 0.1	4.7 \pm 0.3 ^A	2.5 \pm 0.1 ^B
	Lactate	2.7 \pm 0.4	2.6 \pm 0.5	3.0 \pm 0.5	2.3 \pm 0.4	3.2 \pm 0.7	2.5 \pm 0.3	7.0 \pm 0.8 ^A	0.8 \pm 0.1 ^B

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